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Role of nitric oxide increase on induced programmed cell death during early stages of rat liver regeneration ☆

María Teresa Ronco[a], María de Luján Alvarez[a], Juan Alberto Monti[a], María Cristina Carrillo[a], Gerardo Bruno Pisani[b], María Cristina Lugano[b], Cristina Ester Carnovale[a,*]

[a] *Instituto de Fisiología Experimental-Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Suipacha 570-2000 Rosario, Argentina*
[b] *Area de Morfología, Facultad de Ciencias Bioquímicas y Farmacéuticas (Universidad Nacional de Rosario), Argentina*

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Abstract

We analysed the possible cellular mechanism involved in the NO action in the balance between apoptosis and cell proliferation in liver regeneration process. We determined p53, proapoptotic protein Bax, antiapoptotic Bcl-x_L, proliferating cell nuclear antigen (PCNA) and apoptotic index at the early stages of regenerative process after NO increase by lipopolysaccharide-induction (LPS) of inducible-type nitric oxide synthase (iNOS) and by direct NO donor (sodium nitroprusside, SNP). Male Wistar rats were randomised in four experimental groups: sham operated control (Sh), partial hepatectomised control (PH-C), partial hepatectomised pretreated with LPS (2 mg/kg body weight, i.p.) (PH-LPS), and partial hepatectomised pretreated with SNP (2.5 mg/kg body weight, i.v. at a rate of 1 ml/h) (PH-SNP). Animals were killed 5 h post-surgery. Hepatic cytosolic iNOS showed an increase of 34% in PH-C animals with respect to Sh, and LPS-treatment increased iNOS protein levels 30% compared with PH-C. Bax and p53 protein levels showed significant increases in LPS- and SNP-treated hepatectomised rats with respect to PH-C. The apoptotic indexes were increased 75% in both, PH-LPS and PH-SNP rats versus PH-C. The increase of NO did not show any change in the proliferation process. These results suggest that NO is involved in apoptosis via p53 and Bax proteins after PH, showing a tightly regulated growth process in liver regeneration.

Keywords: Nitric oxide; Proliferative process; Apoptosis; Partial hepatectomy; Proapoptotic protein

1. Introduction

The liver has the ability to regenerate following partial hepatectomy (PH). The mechanism regulating this process is complex and incompletely understood and involves a complex interplay of many cellular events [1]. The cell death/proliferation balance is of the most importance in the regenerative process [2]. One change in the immediate hours following PH is the in vivo induction of nitric oxide synthase and the release of nitric oxide (NO) [3–5].

NO is a highly reactive oxidant produced by liver parenchymal and nonparenchymal cells from L-arginine, via an inducible form of nitric oxide synthase (iNOS or NOS2) [4,6]. iNOS is up-regulated in the liver under a number of conditions, including endotoxemia, hemorrhagic shock, ischemia-reperfusion, sepsis, infection, hepatitis, ozone exposure, and liver regeneration [7–10]. Once iNOS is expressed, large amounts of NO are generated in the liver in a substained fashion, playing pivotal roles in a large number of metabolic and immune processes [11]. In some systems the progressive intra- or extracellular generation of NO has been suggested to cause apoptosis [12]. Apoptosis, also called programmed cell death, is an evolutionary conserved phenomenon, which regulates normal cellular turnover [13]. When up-regulation of iNOS occurs, it has been shown that Bcl-2 gene acts as a modulator of the oxidant-antioxidant status of the cell [12]. Members of Bcl-2 family include proteins that prevent cells from apoptosis (Bcl-2, Bcl-x_L, Bcl-w) while others can promote programmed cell death (Bax, Bad, Bak, Bcl-x_S) [2]. On the

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* Corresponding author. Fax: +54-341-4399473.
E-mail address: ifise1@citynet.net.ar (C.E. Carnovale).

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other hand, p53 acts as checkpoint control of the cell cycle, permitting the repair of damaged DNA. The blockage in G1/S transition that results from p53 activation has been suggested to cause apoptosis in the case of severe DNA damage [14].

In a previous work, we found a marked decrease in the peak of DNA synthesis in hepatectomised animals when NO production was diminished by two NOS inhibitors (aminoguanidine or N^6-monnomethyl-L-arginine) [5]. An open question underlying the controversial role of NO is the influence of an increase of NO in the regenerative process. In order to investigate the precise role that NO plays in the balance between apoptosis and cell proliferation, we studied the effect of increase of NO, produced by iNOS induction or by the administration of a direct NO donor, on the expression of proapoptotic proteins (Bax, p53) and antiapoptotic protein (Bcl-x_L), as well as the proliferative and apoptotic indices.

2. Materials and methods

2.1. Animals and surgical procedures

Male Wistar rats weighing 360 to 400 g were housed two per cage and maintained under a 12-h light/dark period. Rats were fed ad libitum with a normal standard diet and water. All the experimental protocols were performed according to the "Guide for the Care and Use of Laboratory Animals" (National Institutes of Health, Publication no. 86-23, revised 1985).

Two-thirds hepatectomy (PH) consisted of removal of the central and left lateral lobes as was originally described by Higgins and Anderson [15]. Sham operated (Sh, simulated surgery) and PH animals were laparotomised under pentobarbital anesthesia (50 mg/Kg body wt., i.p.). Animals were killed 5 and 24 h after surgery. To avoid variations due to circadian rhythms, the time to perform the surgery had to be fixed in order to sacrifice the animals always at the same time of the day (between 10:00 and 12:00 h).

At the time of sacrifice, all rats were bled through a cardiac puncture after pentobarbital anesthesia and livers were removed. Pieces of liver tissue were processed for immunohistochemical studies. The remaining liver tissue was frozen in liquid nitrogen and stored at −70 °C until the analytical assays.

2.2. Induction of nitric oxide synthase

As iNOS inductor we used lipopolysaccharide (LPS). Animals were randomised in two groups and received isotonic saline (Control) and LPS 2 mg/kg body wt. (LPS). Each group was subdivided into Sham and PH. The animals were injected with LPS 1 h before PH and were killed 5 h after surgery [16].

2.3. Increase of nitric oxide by sodium nitroprusside

As direct NO donor we used sodium nitroprusside (SNP). SNP (Merck) was dissolved and diluted in 0.9% of NaCl. Care was taken to protect SNP solutions from light due to its light sensitivity. Animals were randomised in two groups and received isotonic saline (Control) infusion during 30 min at a rate of 1 ml/h, and SNP 2.5 mg/body wt. infusion during 30 min at a rate of 1 ml/h. Each group was subdivided into Sham and PH. After this period, rats were sacrificed [17].

2.4. Analytical assays

2.4.1. Nitrate determination

NO formation was measured indirectly assaying nitrate, one of the stable end products of NO oxidation. An assay based on the conversion of nitrate to nitrite by reduced nicotinamide–adenine dinucleotide phosphate (NADPH) in the presence of the nitrate reductase enzyme (EC 1.6.6.2) was used [18]. The amount of NADPH oxidised during the reaction was stoichiometrically equal with the amount of nitrate. The decrease in NADPH was measured by means of its absorbance at 340 nm.

2.4.2. Western blot analysis: iNOS, Bax, Bcl-x_L and p53

For iNOS and p53 detection, liver tissue lysates were prepared by homogenization of frozen tissues in 3 volumes of lysating RIPA buffer containing PBS, 1% Triton, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 1 μg/ml aprotinin. After 30 min of incubation at 0 °C and three freeze–thaw cycles, lysates were cleared by centrifugation at 15 000 rpm for 30 min, and supernatants were kept at −70 °C. For the preparation of mitochondria-enriched fractions, liver tissues were homogenised in 4 volumes of 300 mmol/l sucrose with protease inhibitors. Homogenates were centrifuged at $1000 \times g$ to remove unbroken cells, nuclei and heavy membranes. Mitochondria enriched fractions were then obtained by centrifugation at $3000 \times g$ at 4 °C for 15 min [19]. Proteins were quantified according to Lowry et al. [20]. For iNOS analysis, 100 μg of protein was separated by 7.5% SDS-polyacrylamide gel electrophoresis and transferred to polyvinyl difluoride membranes (PVDF). For p53, Bax and Bcl-x_L detection, 50 μg of protein was subjected to 12% SDS-polyacrylamide gel electrophoresis and transferred to PVDF (PerkinElmer Life Sciences, Boston, MA, USA). After blocking, blots were incubated overnight at 4 °C with either polyclonal anti-iNOS antibody (1:2000, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-mouse Bax or anti-human Bcl-x_L or anti-mammalian p53 antibodies (1:500, Santa Cruz Biotechnology). The membranes were then incubated with either anti-rabbit or anti-mouse Ig G-peroxidase conjugates (1:5000, Amersham Life Science) and bands were detected by enhanced chemiluminescence detection (ECL; Amer-

sham Pharmacia Biotech). Autoradiographs were obtained by exposing polyvinyl difluoride membranes to Kodak XAR film, and the bands were quantitated by densitometry (Shimadzu CS-9000).

2.4.3. Preparation of tissues for histological studies

Liver slices were fixed in 10% v/v formalin solution and embedded in low melting paraffin. Sections of tissue were deparaffinised in xylene and alcohol and then rehydrated in deionized water. Immunohistochemical staining procedures were then performed [21].

2.5. Evaluation of cell death:

2.5.1. Determination of apoptotic index (AI)

Quantitative analysis of apoptosis was performed by in situ specific labeling of fragmented DNA using a modified terminal deoxynucleotidyl transferase (Tdt) mediated biotin-deoxyuridine triphosphate nick-end labeling (TUNEL) method [22] (Promega, Madison, WI, USA). Apoptotic cells were counted only if they were TUNEL-positive and displayed hallmark characteristics of apoptosis. An apoptotic index (AI) was calculated for each sample by counting the number of positively stained hepatocyte nuclei divided by the total number of hepatocytes and expressed as percentage. The number of apoptotic hepatocytes was assessed by systematically scoring at least 6000 hepatocytes per slide at a magnification of 400 ×.

To corroborate the incidence of apoptotic bodies, serial sections were stained with hematoxylin–eosin.

2.6. Evaluation of proliferation process

2.6.1. Determination of proliferative index (PI)

For this evaluation, we performed the studies 24 h after surgery. It is known that the maximum proliferation level is observed at 24 h after PH and decreases toward 48 h, although maintaining increased with respect to the Sh-Control value. Sections of slides were examined by immunohistochemical staining with anti-PCNA (proliferating cell nuclear antigen) antibodies (Santa Cruz Bio technology). PCNA was visualised by the method of Greenwell et al. [23] as primary antibody. Ten random fields of liver sections (400 × magnification) were evaluated and scored. All PCNA-positive cells in G1, S, M and G2 phases were judged as proliferating cells and scored per 1000 hepatocytes.

2.6.2. DNA synthesis

For this evaluation, we performed the studies 24 h after the surgery. [^{3}H]-Thymidine (New Life Sciences, Boston, USA) (10 μCi/200 g body wt.) was injected intraperitoneally, and rats were sacrificed 1 h after injection. Liver tissues were immediately excised and washed with ice-cold physiological saline. The incorporation of radio activity into the acid-insoluble fraction was measured by a Liquid Scintillation Counter (1214 Rack Beta, Pharmacia, Wallac OY, Finland) and was expressed as dpm/mg DNA [24].

2.7. Statistical analysis

Results were expressed as mean ± S.E. Significance in differences was tested by one-way ANOVA, followed by Tukey's test. Differences were considered significant when the P value was <0.05. Correlations were examined by Spearman rank linear regression.

3. Results

3.1. Cytosolic nitrate level

We examined the concentration of nitrates in hepatic cytosol as a measurement of NO production. LPS and SNP Sham treated animals showed an increase of less than 15% in nitrates levels with respect to Sham control animals, which does not represent statistical difference. In concordance with other authors, Fig. 1 shows a clear increase (80%) in the content of nitrates in hepatic cytosol 5 h post-hepatectomy [4]. Both treatments, LPS and SNP, which were utilized as a

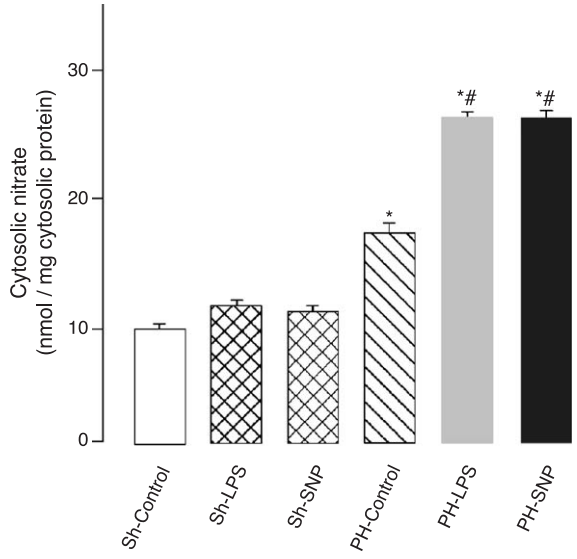

Fig. 1. Production of nitrate, a metabolic product of NO, measured in hepatic cytosol expressed as nmol/mg protein at 5 h post-surgery. Sh, Sham operated; Sh-LPS, treated with LPS 2 mg/body wt.; Sh-SNP, treated with SNP 2.5 mg/body wt.; PH-C, partial hepatectomy control; PH-LPS, PH-treated with LPS 2 mg/body wt.; and PH-SNP, PH-treated with SNP 2.5 mg/body wt. Sh-LPS and Sh-SNP treated animals show no statistically significant difference with respect to Sh-Control group. PH-C corresponding to both treatment (LPS vehicle i.p. and SNP vehicle i.v. during 30 min at a rate of 1 ml/h) showed no statistically difference between them. Results show means ± S.E. of six rats. *Significant difference vs. Sh-Control ($P<0.05$). #Significant difference vs. PH-Control group ($P<0.05$).

tool for increasing NO levels, effectively produced an increase of 56% in nirate levels compared with partial hepatectomised control (PH-C).

The aim of this work was to increase NO levels in hepatectomised rats and, as Sham treated animals did not show any difference with respect to Sham control animals, the results were analysed with respect to Sham-control.

3.2. Nitric oxide synthase

We examined iNOS protein expression in hepatic cytosolic fraction 5 h post-hepatectomy with and without LPS (iNOS inductor) treatment (Fig. 2). The treatment with LPS produced an increase of 30% in iNOS protein expression. We observed that, after LPS treatment, nitrate levels increase in parallel with iNOS protein expression. In this sense, we observed a linear relationship between the two parameters ($r = 0.843$, $P = 0.0052$, $n = 10$).

3.3. Bax, Bcl-x_L and p53

We examined the expression of Bax and Bcl-x_L proteins by Western blot analysis 5 h post-PH. Bax and Bcl-x_L are members of the Bcl-2 protein family which play a major role as regulators of the apoptotic process: while Bax promotes apoptosis, Bcl-x_L protects cells from programmed cell death [25].

We analyzed the expression of Bax protein in liver mitochondrial fraction. Bax showed an increase of 56% in the liver mitochondrial fraction at 5 h after PH, in animals treated with LPS, with respect to their PH-C animals (Fig. 3). Moreover, SNP increased Bax protein level in 45% with respect to PH-C (Fig. 3).

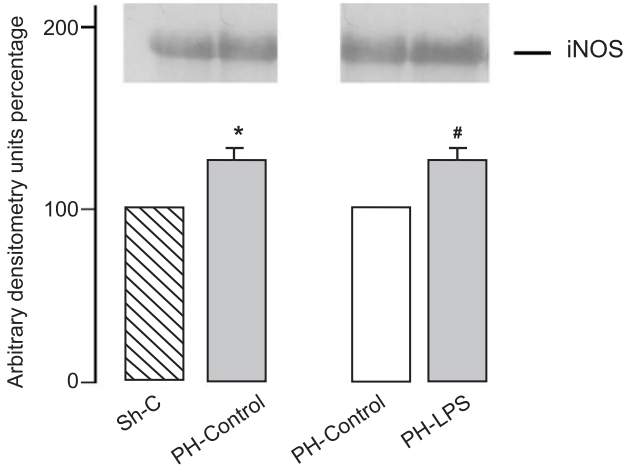

Fig. 2. Immunoblot for iNOS protein performed at 5 h post-surgery was determined as described in Materials and methods. Lane 1: Sham, Lanes 2 and 3: PH-Control, Lane 4: PH-LPS. Specific bands corresponding to iNOS were scanned and quantitated and the data are presented graphically. PH and PH-LPS 2 mg/body wt. Results show the mean ± S.E. of six rats. *Significant difference vs. Sham ($P < 0.05$). #Significance difference vs. PH-Control group ($P < 0.05$).

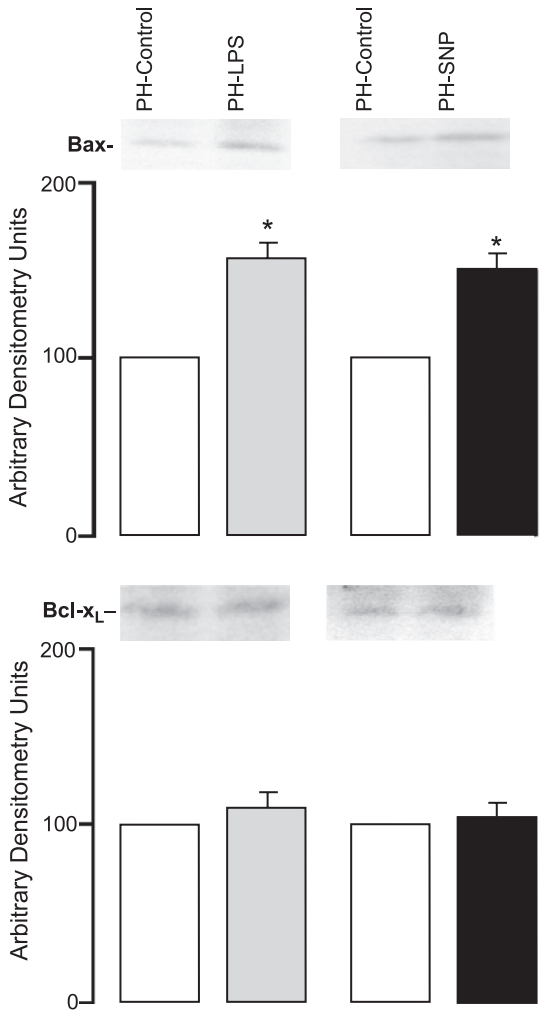

Fig. 3. Immunoblot analysis of Bax and Bcl-x_L protein expression in liver mitochondrial fraction at 5 h post-PH, with and without treatment. Lanes 1 and 3: PH-Control, Lane 2: PH-LPS, Lane 3: PH-SNP. The accompanying bars represent the densitometry expressed in percent from five separate animals sets. Considering PH-Control as 100%. Bax: PH-LPS and PH-SNP show an increase of 56% and 45% with respect to PH-Control, respectively. Bcl-x_L: PH-LPS and PH-SNP show no difference with respect to PH-Control. *Statistically significance against PH-Control.

Mitochondrial Bcl-x_L levels of LPS and SNP treated animals did not show difference with respect to corresponding PH-C (Fig. 3).

In order to evaluate the role of p53, we analyzed the expression of this protein in total liver lysates. Five hours post-PH, both treatments, LPS and SNP, produced an increase of 40% in p53 protein levels (Fig. 4).

3.4. Apoptotic index

Apoptotic cells were identified at 5 h after PH. Table 1 shows the AI in each experimental group. Apoptosis was induced in treated-animals at 5 h post-PH with both treatments, LPS and SNP.

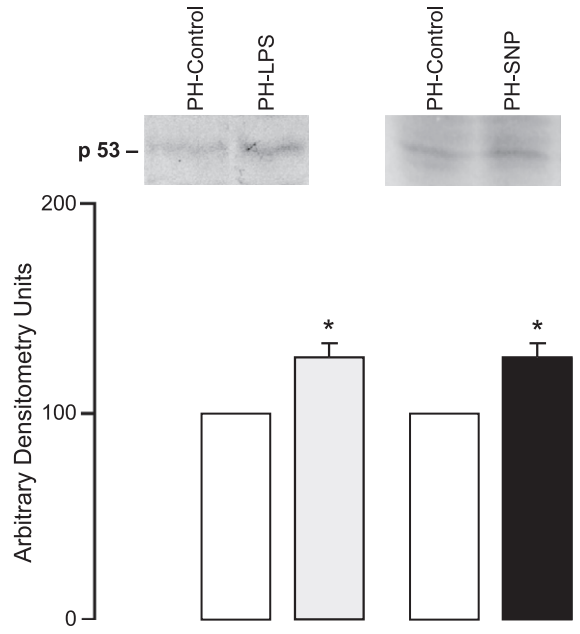

Fig. 4. Immunoblot analysis of p53 protein expression in liver total lysate of 5 h post-PH. Lanes 1 and 3: PH-Control, Lane 2: PH-LPS, Lane 3: PH-SNP. Quantitation of immunoblot bands was performed by densitometry considering PH-Control as 100%. p53 showed an increase of 40% in both treatments with respect to their PH-Control. Results show the mean ± S.E. of six rats. *Significance difference vs. the corresponding PH-Control.

3.5. Regenerative process

The regenerative process that follows PH was monitored by the time course of [^{3}H]-thymidine incorporation into DNA in the remnant tissue. In concordance with several reports [26–28], the rate of DNA synthesis peaks 24 h after hepatectomy maintaining a significant difference with respect to Sham animals. Both treatments did not produce any change in the rate of DNA synthesis 24 h post-hepatectomy compared with PH-C (Table 2). In order to evaluate if NO forwards the rate of DNA synthesis, we studied the time course of [^{3}H]-thymidine incorporation into DNA in the liver of animals sacrificed 19 h post-PH. The treatments did not produce any difference with respect to PH-C animals (data not shown). PI also showed an increase at 24 h after hepatectomy. The treatments with LPS and SNP did not produce any change in the PI, 24 h post-PH (Table 3).

Table 1
Apoptotic index (AI)

Sh-C	PH-C	PH-LPS	PH-SNP
1.00 ± 0.10	1.50 ± 0.10$^{\#}$	2.80 ± 0.40$^{\#,*}$	2.44 ± 0.10$^{\#,*}$

AI was expressed as percentage of apoptotic cells scored per 100 hepatocytes per slide at a magnification of 400×. All values are expressed as means ± S.E. of six rats per group.

$^{\#}$ $P<0.05$ vs. Sh-C.

* $P<0.05$ vs. PH-C.

Table 2
[^{3}H]-Thymidine incorporation to DNA

Sh-C	PH-C	PH-LPS	PH-SNP
5.1 ± 1.2	41.3 ± 3.1*	41.2 ± 3.2*	41.0 ± 3.0*

The incorporation of radioactivity into the acid-insoluble fraction was expressed as 10^4 dpm/mg DNA. All values represent mean ± S.E. of six animals per group.

* $P<0.05$ vs. Sh-Control.

3.6. Regression coefficient values

The regression coefficient values were obtained from PH-C and pretreated (LPS and SNP) PH animals. A direct linear relationship between cytosolic nitrate level and Bax protein was obtained ($r=0.842$; $P<0.004$; $n=10$). In the same way, a direct relationship was obtained between cytosolic nitrate level and both, p53 expression ($r=0.795$, $P<0.018$, $n=10$) and apoptotic index ($r=0.723$, $P<0.004$, $n=10$).

4. Discussion

The study of liver regeneration is well established as a research area of considerable interest both from the pathophysiological and experimental points of view [1,29]. One of the models to accomplish the regenerative process is through the surgical resection of a major portion (65%) of liver. In this case, the remnant liver starts a series of time responses intended first to favour cell growth and then to stop the hepatocyte proliferation once the liver function is fully restored [30].

Earlier studies had shown that in the immediate hours following PH exist the induction of iNOS and the release of NO in vivo [4,31]. The importance of NO in regenerative process is suggested by the finding that liver regeneration is defective in iNOS knock-out mice [32]. In accordance, previous experiments in our laboratory had shown that NO is involved in regenerative response because inhibition of iNOS by a specific inhibitor (L-NAME) and a nonspecific inhibitor (aminoguanidine) resulted in a decrease of hepatocyte proliferation after PH [4]. Several observations suggest that NO release after PH is necessary to achieve the normal regenerative process [33,34].

Although NO is a very important messenger molecule, in certain cellular types it can induce apoptosis [12]. In

Table 3
Proliferation index (PI)

Sh-C	PH-C	PH-LPS	PH-SNP
3.3 ± 1.3	21.4 ± 1.5*	24.2 ± 1.2*	26.6 ± 1.0*

PI was expressed as proliferating cells per 1000 hepatocytes evaluated in 10 random fields of liver sections (400× magnification). All values represent mean ± S.E. of six animals per group.

* $P<0.05$ vs. Sh-Control.

accordance with this controversial role of NO, and in order to get insight about the role of NO in liver regeneration, in this work we increased NO levels with LPS, a potent iNOS inductor which up-regulates iNOS in multiple cell types including hepatocytes, and also with a direct NO donor, i.e. SNP [17,35].

It is known that Bax protein promotes cell death via homodimerization, whereas heterodimerization with Bcl-x_L results in cell survival [2]. In a previous work, our findings indicate that during the first hours post PH, Bax protein levels were elevated [36]. After iNOS induction and SNP treatment, Bax protein levels were increased in hepatectomised rats with respect to PH-C groups, showing a direct linear relationship with NO levels. On the other hand, Bcl-x_L did not show any difference between the treated and control groups.

Expression of wild-type p53, a tumor suppressor gene, seems to be closely linked with apoptosis caused by most of DNA-damage agents [14,37]. At the time studied, both treatments increased p53 protein levels in PH animals. And we demonstrated that there exists a correlation between cytosolic nitrate levels and p53 protein levels. Besides, the treatment with iNOS inductor and NO donor increased the AI, showing a direct linear relationship between NO and AI. These results indicate that induction of NOS and concomitant NO generation or NO donor result in cell death by apoptosis. Whether a cell undergoes apoptosis appears to be due to a shift in the balance between anti- and proapoptotic factors in favor of the proapoptotic. Moreover, our results suggest that increase of NO can impair this balance by augmentation of proapoptotic proteins, Bax and p53.

On the other hand, our results indicate that no change exists in the proliferation of resting liver after NOS induction nor with NO donor demonstrating that an over-increase of NO does not modify the proliferative process (Tables 2 and 3).

These results suggest that NO may be involved in the vascular readaptation after PH, favoring a general permeability throughout the organ for the different growth factors. In this regard, several reports confirm the important role of NO in the process of vascularization, angiogenesis and permeabilization of tissues [5,32,38,39].

Finally, our results propose a role for NO in the tight regulation of liver regeneration process post-PH, which produces a marked increase in cellular apoptosis and any change in the proliferation process, suggesting that NO might be involved in the process of vascularization of remnant liver. Thus, the NO released after PH, is necessary and enough to achieve a normal regenerative process.

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